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ORIGINAL ARTICLE

Repeated isolation of virulent Newcastle disease viruses of sub-genotype VIId from backyard chickens in Bulgaria and Ukraine between 2002 and 2013

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Abstract Here, we report the circulation of highly related virulent Newcastle disease viruses (NDV) in Bulgaria and Ukraine from 2002 until 2013. All of these NDV isolates have the same virulence-associated cleavage site ("$_{113}$RQKR↓F$_{117}$"), and selected ones have intracerebral pathogenicity index values ranging from 1.61 to 1.96. These isolates are most closely related to viruses circulating in Eastern Europe, followed by viruses isolated in Asia during the same period of time. Interestingly, the majority of the viruses were isolated from backyard poultry, suggesting the possibility of a "domestic" or "urban" cycle of maintenance. The molecular characterization of the nucleotide sequence of the complete fusion protein gene of the studied viruses suggests continued circulation of virulent NDV of sub-genotype VIId in Eastern Europe, with occasional introductions from Asia. Furthermore, the high level of genetic similarity among those isolates suggests that the NDV isolates of sub-genotype VIId from Bulgaria and Ukraine may have been part of a broader epizootic process in Eastern Europe rather than separate introductions from Asia or Africa. The continuous monitoring of backyard poultry flocks for the presence of circulating virulent NDV strains will allow early identification of Newcastle disease outbreaks.

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Introduction

Newcastle disease virus (NDV) is one of the most important pathogens of poultry worldwide [2, 26]. Newcastle disease (ND) is caused by virulent strains of avian paramyxovirus 1 (APMV-1, synonymous to NDV). APMV-1 belongs to the family *Paramyxoviridae*, genus *Avulavirus* of the order *Mononegavirales* [23]. The disease devastates poultry flocks with up to 100 % mortality and also causes significant economic losses from trade restrictions imposed on countries and regions where outbreaks have occurred [6, 26]. Based on genomic analysis, APMV-1 isolates are classified into two main evolutionary related groups – class I and class II [11]. Employing the coding sequence of the fusion protein gene (F gene) or the complete genome sequence, NDV isolates can be further classified into genotypes [13]. Currently, there are 18 genotypes in class II (genotypes I to XVIII) and a single genotype in class I (genotype 1), with some genotypes further divided into sub-genotypes [9, 12, 13, 41, 42].

Viruses of class II genotype VII are responsible for the fourth ND panzootic, which began around 1985 in Southeast Asia, spread to Africa, Europe and South America, and continues today [16, 21, 22, 26, 37, 51]. Since 2011, reports have documented that some of the newly emerged viruses of genotype VII can cause mortality in poorly vaccinated

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poultry [39, 50]. Furthermore, viruses of genotype VII have been frequently isolated from wild and domestic waterfowl [17, 20, 46], with documented cases of clinical disease in geese, which have been shown previously to be resistant to morbidity [17, 26, 48].

Bulgaria and Ukraine are wintering sites for many wild-bird species and are also located within the largest wild-bird migration system – the Black Sea-Mediterranean flyway. Some areas of this flyway overlap with other major migration systems (e.g., Central and East Asian-Australasian flyways, West Asian-East African flyway, East Atlantic flyway) [31]. Additionally, Bulgaria and Ukraine are geographically located on main trade routes between Asia and Europe. The continuous expansion of the poultry industry worldwide and international trade of poultry products [38] is reflected in Bulgaria and Ukraine with these two countries having well-developed poultry farming industries. As a result of established good vaccination practices, ND outbreaks on industrial poultry farms have not occurred for almost a decade in those countries. However, the backyard poultry population is still considerably large and widely distributed. Not all backyard birds are vaccinated, and biosecurity management is scarce or non-existent. This provides opportunity for virulent ND viruses to infect and cause clinical disease after the introduction in these birds [14, 29].

Previous studies of virulent NDV (vNDV) isolates from Bulgaria and Ukraine have been performed employing mainly serological and conventional virological approaches. Molecular analyses have also been performed, but these have been based on limited genetic information (partial F gene sequences) or, in some cases, targeting low-virulence NDV from wild birds [1, 10, 16, 18, 29, 34]. Here, we report the circulation of highly related virulent viruses from 2002 until 2013 in Bulgaria and Ukraine and of one isolate from Russia. The epidemiology of ND was evaluated on the basis of phylogenetic analysis of the complete coding sequence of the fusion protein gene of these isolates.

Materials and methods

Sample collection and virus isolation

Information for the isolates studied in this work, including location, bird species, presence of clinical signs, husbandry practice, vaccination history, and type of samples, is presented in Table 1. For the samples from Bulgaria, sampled swabs were placed in tubes with the following transport medium: medium 199 supplemented with 10,000 IU of penicillin, 10 mg of streptomycin, 0.250 mg of gentamicin and 1 mg of oxytetracycline per ml, chilled at 4 °C and transported to the lab for immediate testing. Swab medium (0.2 ml) from each sample was inoculated into five 9 to 11-day-old embryonating chicken eggs (ECEs) from NDV- and AIV-free layers using standard methods as described previously [3]. For the Ukrainian samples, immediately after sampling, the swabs were placed in tubes with transport medium (Hank's balanced salt solution containing 0.5 % lactalbumin, 10 % glycerol, and 200 U penicillin, 0.200 mg streptomycin, 100 U polymyxin, 0.250 mg gentamicin, and 50 U nystatin per ml). Organ suspensions (10 % w/v) were also prepared using the same medium. Samples were chilled at 4 °C, transported to the lab and stored at -70 °C for 1 to 4 weeks until analyzed. Swab medium or suspension supernatant (0.2 ml) from each sample was inoculated into five 9 to 11-day-old specific-pathogen-free (SPF) embryonating chicken eggs using standard methods as described previously [3]. For the Russian sample, the virus isolation procedure was similar to the one described for the Ukrainian sample with the difference that NDV- and AIV-free ECEs were used instead of SPF. For all samples, the allantoic fluids from inoculated ECEs were harvested and subjected to a hemagglutination assay (HA) using the microtiter method. All of the hemagglutinating samples were confirmed to be APMV-1 by hemagglutination inhibition (HI) assay [32].

Virus propagation

Twenty-one isolates (Table 1) were submitted to the Southeast Poultry Research Laboratory of the USDA in Athens, GA, USA. Viruses were propagated in 9 to 11-day-old SPF embryonating chicken eggs from the SEPRL SPF white leghorn flock [3].

Intracerebral pathogenicity index (ICPI) assay

An intracerebral pathogenicity index (ICPI) assay was conducted on 14 viruses (selection was based on phylogenetic analysis) following established procedures [32]. The ICPI test for isolate chicken/Ukraine/Ivano-Frankivsk/58/2007 was performed in Ukraine following the same procedures.

RNA isolation, PCR amplification and sequencing

RNA from each isolate was extracted from allantoic fluids using TRIzol LS (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. One-step reverse transcription PCR (SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase, Life Technologies, Carlsbad, CA, USA) was used to convert and amplify the extracted RNA as described previously [28] using primers described by Miller et al. (4008F/4994R; 4715F/5637R; 5410F/6332R) [27]. All PCR products were subjected to

Table 1 Background information data for 21 virulent NDV isolates recovered in Bulgaria, Ukraine and Russia between 2002 and 2013

Species	Country	Isolate	Year of isolation	Sample type	Clinical signs[a]	Ecotype	Vaccination history	ICPI[b]	Accession number
Chicken	Bulgaria	Yuper	2006	CS[c], OS[d]	Yes	Backyard	No	1.93	KU295450
Chicken	Bulgaria	Vodniantsi	2006	CS, OS	Yes	Backyard	No	1.88	KU710269
Chicken	Bulgaria	Moravitsa	2007	CS, OS	Yes	Backyard	No	1.96	KU295451
Chicken	Bulgaria	Vidno	2007	CS, OS	Yes	Backyard	No	1.94	KU295452
Chicken	Bulgaria	Smolianovtsi	2007	CS, OS	Yes	Backyard	No	1.88	KU710270
Chicken	Bulgaria	Kravoder	2007	CS, OS	Yes	Backyard	No	1.88	KU710271
Chicken	Bulgaria	Liliashka Mogila	2008	CS, OS	Yes	Backyard	No	1.88	KU710272
Chicken	Bulgaria	Kardam	2008	CS, OS	Yes	Backyard	No	1.86	KU710273
Chicken	Bulgaria	Kazatsite	2008	CS, OS	Yes	Backyard	No	N/P[e]	KU710274
Chicken	Bulgaria	Vozhdovo	2009	CS, OS	Yes	Backyard	No	1.89	KU710275
Chicken	Bulgaria	Mamarchevo	2009	CS, OS	Yes	Backyard	No	1.88	KU710276
Chicken	Ukraine	Lypova Dolyna	2002	CS, OS, organs	Yes	Backyard	No	N/P	KU710278
Chicken	Ukraine	Lyubotyn	2003	CS, OS, organs	Yes	Small farm	Yes	1.85	KU295454
Chicken	Ukraine	Lugansk	2003	CS, OS, organs	Yes	Backyard	No	N/P	KU710279
Chicken	Ukraine	Tsyrkuny	2003	CS, OS, organs	Yes	Backyard	No	N/P	KU710280
Chicken	Ukraine	Pokotylivka	2003	CS, OS, organs	Yes	Backyard	No	N/P	KU710281
Chicken	Ukraine	Ivano-Frankivsk/58	2007	CS, OS, organs	Yes	Backyard	No	1.61	KJ914673
Chicken	Ukraine	Kharkiv/66	2007	CS, OS, organs	Yes	Backyard	No	1.83	KU295453
Pigeon	Ukraine	Simferopol/2-26	2011	organs	Found dead	wild bird	N/A[f]	N/P	KU710277
Chicken	Ukraine	Bashtanivske/20-02	2013	CS, OS, organs	Yes	Backyard	Yes	1.86	KU295455
Mallard	Russia	Adygea/927	2007	organs	Found dead	wild bird	N/A	N/P	KU726619

[a] Neurological, respiratory and enteric clinical signs were observed with differences in severity and number of affected birds

[b] ICPI = intracerebral pathogenicity index

[c] CS = cloacal swab

[d] OS = oropharyngeal swab

[e] N/P = not performed

[f] N/A = not applicable

electrophoresis in a 1% agarose gel (0.5X TBE). The DNA bands were excised and purified using a QuickClean II Gel Extraction Kit (GenScript, Piscataway, NJ, USA). Nucleotide sequencing and assembly were performed as described previously by Miller et al. [28].

Collection of sequences

Complete fusion protein gene (F gene) coding sequences (n = 1458) of all available class II NDV isolates were downloaded from GenBank (National Center for Biotechnology Information) (available as of January 2015, http://www.ncbi.nlm.nih.gov/genbank) and aligned using ClustalW [45].

Evolutionary and phylogenetic analysis

Phylogenetic analysis was performed using MEGA6 software (MEGA, version 6) [44]. Preliminary analysis was performed to infer the evolutionary history with 1458 sequences available in GenBank (data not shown). A smaller group (including the most closely related viruses, n = 82) (Table S1) was parsed from the initial dataset and further analyzed. Determination of the best-fit substitution model was performed using MEGA6, and the goodness-of-fit for each model was measured by corrected Akaike information criterion (AICc) and Bayesian information criterion (BIC) [44]. The final tree was constructed using the maximum-likelihood method based on the general time-reversible model as implemented in MEGA 6, with 500 bootstrap replicates [30]. For all analyses, the codon positions included were 1st + 2nd + 3rd + noncoding, and all positions containing gaps and missing data were eliminated. The Roman numerals presented for each sequence in the phylogenetic tree represent the genotype of the isolate, followed by the GenBank accession number, host name (if available), country of isolation, strain designation, and year of isolation.

The estimates of average evolutionary distances were inferred using MEGA6. Analyses were conducted using the

maximum composite likelihood model [43]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 4). Previously described criteria [13] based on the phylogenetic topology and evolutionary distances between different taxonomic groups were used to determine genotypes and sub-genotypes.

Accession numbers

The complete F gene sequences (n = 21) of virulent NDV obtained in this study were submitted to GenBank and are available under the accession numbers KJ914673, KU295450 to KU295455, KU710269 to KU710281 and KU726619.

Results

Virus recovery and ICPI test

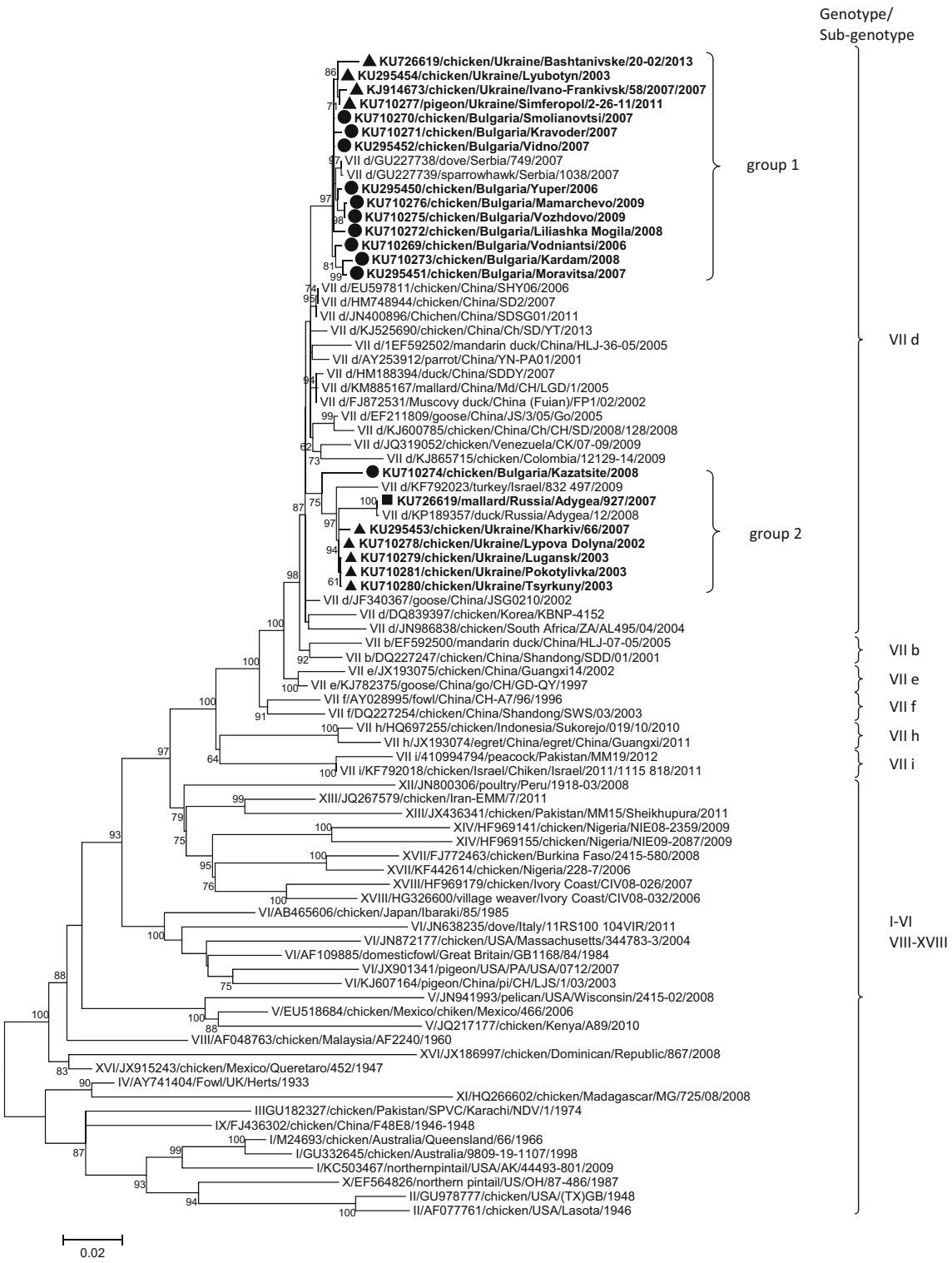
Twenty-one NDV isolates were studied in the present work. In Bulgaria and Ukraine, national monitoring programs (active surveillance) are implemented, and both industrial and backyard poultry are monitored for the presence of virulent Newcastle disease viruses. Eleven of the viruses in this study were isolated in Bulgaria and were representative for all 33 NDV outbreaks (sub-genotype VIId) that were identified during the described period. Selection was done based on phylogenetic analysis of the 374-nucleotide region of the fusion protein gene (data not shown). However, our data on the prevalence of NDV genotypes in Ukraine between 2002 and 2013 is limited to the viruses studied here. Most of the samples in the present study (n = 18) were recovered from unvaccinated backyard chickens that showed respiratory, neurological and/or enteric signs. The viruses were recovered from six regions in Bulgaria, six regions in Ukraine and one region in Russia in 2006–2009, 2002–2013 and 2007, respectively (Table 1). Biological assessment of the pathogenicity of selected isolates was performed by ICPI test in 1-day-old SPF chickens. All of the viruses tested using the ICPI assay yielded values ranging from 1.61 to 1.93 (Table 1), which characterizes them as virulent NDV based on the OIE standards [32] and is in agreement with the observed clinical signs. ICPI values above 1.5 are indicative of velogenic NDV [3].

Nucleotide sequencing and distance analysis

To determine the genetic characteristics of the NDV isolated in Bulgaria, Ukraine and Russia, we performed sequencing and distance analysis of these isolates. The sequencing analysis predicted fusion protein cleavage sites that contained three basic amino acids at positions 113–116 and a phenylalanine residue at position 117 ("$_{113}$-RQKR↓F$_{117}$") in all isolates. Such a cleavage site is specific for virulent viruses based on criteria utilized by OIE to assess virulence of NDV isolates [32], and these results are in agreement with the data obtained in the ICPI test.

All isolates were analyzed in order to determine the evolutionary distances among them and between them and other ND viruses. Phylogenetic analysis of the complete fusion coding regions classified all of the isolates studied here as class II sub-genotype VIId. Their genetic distance when compared to the rest of the sub-genotype VIId isolates was in the range of 1.7 to 2.9 %. Fourteen of the isolates obtained in Bulgaria and Ukraine between 2003 and 2013 were very similar among themselves, and the mean nucleotide sequence identity within this group was 99.4 %. The nearest related isolates (99.3-99.7 % genetic identity) were dove/Serbia/749/2007 and sparrowhawk/Serbia/1038/2007. The 14 viruses were also genetically close (98.6-98.7 % genetic identity) to isolates obtained from poultry and wild birds in China between 2002 and 2011 (chicken/China/SHY06/2006, chicken/China/SDSG01/2011, chicken/China/SD2/2007, Muscovy Duck/China/FP1/02/2002 and mallard/China/Md/CH/LGD/1/2005). Seven of the analyzed viruses isolated between 2002 and 2008 (five Ukrainian, one Bulgarian and one Russian, 99 % identity within the group) were more distant and showed 97.3 % nucleotide sequence identity when compared to the rest of Bulgarian and Ukrainian viruses studied here. The five Ukrainian isolates had high genetic similarity among themselves (99.6 %). The isolates that were genetically closest to these seven isolates were duck/Russia/Adygea/12/2008 and turkey/Israel/832 497/2009, with nucleotide sequence identities of 98.7 % and 98.2 %, respectively.

Fig. 1 Phylogenetic analysis based on the complete nucleotide sequence of the fusion protein gene of isolates representing Newcastle disease virus class II. The evolutionary history was inferred by using the maximum-likelihood method based on general time-reversible model with 500 bootstrap replicates as implemented in MEGA 6 [30]. The tree with the highest log likelihood (-17732.1685) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories [+G, parameter = 0.6688]). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 39.7514 %). The percentages of trees in which the associated sequences clustered together are shown below the branches (numbers below 60 are not shown). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.The analysis involved 82 nucleotide sequences with a total of 1651 positions in the final dataset. Bulgarian isolates sequenced in this study are designated with ● in front of the isolate name, Ukrainian isolates are designated with ▲, and the Russian isolate is designated with ■. Evolutionary analysis was conducted in MEGA6 [44] ▶


Genotype/
Sub-genotype
KU726619/chicken/Ukraine/Bashtanivske/20-02/2013
KU295454/chicken/Ukraine/Lyubotyn/2003
KJ914673/chicken/Ukraine/Ivano-Frankivsk/58/2007/2007
KU710277/pigeon/Ukraine/Simferopol/2-26-11/2011
KU710270/chicken/Bulgaria/Smolianovtsi/2007
KU710271/chicken/Bulgaria/Kravoder/2007
KU295452/chicken/Bulgaria/Vidno/2007
VII d/GU227738/dove/Serbia/749/2007
VII d/GU227739/sparrowhawk/Serbia/1038/2007
KU295450/chicken/Bulgaria/Yuper/2006
KU710276/chicken/Bulgaria/Mamarchevo/2009
KU710275/chicken/Bulgaria/Vozhdovo/2009
KU710272/chicken/Bulgaria/Liliashka Mogila/2008
KU710269/chicken/Bulgaria/Vodniantsi/2006
KU710273/chicken/Bulgaria/Kardam/2008
KU295451/chicken/Bulgaria/Moravitsa/2007
group 1
VII d/EU597811/chicken/China/SHY06/2006
VII d/HM748944/chicken/China/SD2/2007
VII d/JN400896/Chichen/China/SDSG01/2011
VII d/KJ525690/chicken/China/Ch/SD/YT/2013
VII d/1EF592502/mandarin duck/China/HLJ-36-05/2005
VII d/AY253912/parrot/China/YN-PA01/2001
VII d/HM188394/duck/China/SDDY/2007
VII d/KM885167/mallard/China/Md/CH/LGD/1/2005
VII d/FJ872531/Muscovy duck/China (Fuian)/FP1/02/2002
VII d/EF211809/goose/China/JS/3/05/Go/2005
VII d/KJ600785/chicken/China/Ch/CH/SD/2008/128/2008
VII d/JQ319052/chicken/Venezuela/CK/07-09/2009
VII d/KJ865715/chicken/Colombia/12129-14/2009
KU710274/chicken/Bulgaria/Kazatsite/2008
VII d/KF792023/turkey/Israel/832 497/2009
KU726619/mallard/Russia/Adygea/927/2007
VII d/KP189357/duck/Russia/Adygea/12/2008
KU295453/chicken/Ukraine/Kharkiv/66/2007
KU710278/chicken/Ukraine/Lypova Dolyna/2002
KU710279/chicken/Ukraine/Lugansk/2003
KU710281/chicken/Ukraine/Pokotylivka/2003
KU710280/chicken/Ukraine/Tsyrkuny/2003
group 2
VII d/JF340367/goose/China/JSG0210/2002
VII d/DQ839397/chicken/Korea/KBNP-4152
VII d/JN986838/chicken/South Africa/ZA/AL495/04/2004
VII d
VII b/EF592500/mandarin duck/China/HLJ-07-05/2005
VII b/DQ227247/chicken/China/Shandong/SDD/01/2001
VII b
VII e/JX193075/chicken/China/Guangxi14/2002
VII e/KJ782375/goose/China/go/CH/GD-QY/1997
VII e
VII f/AY028995/fowl/China/CH-A7/96/1996
VII f/DQ227254/chicken/China/Shandong/SWS/03/2003
VII f
VII h/HQ697255/chicken/Indonesia/Sukorejo/019/10/2010
VII h/JX193074/egret/China/egret/China/Guangxi/2011
VII h
VII i/410994794/peacock/Pakistan/MM19/2012
VII i/KF792018/chicken/Israel/Chiken/Israel/2011/1115 818/2011
VII i
XII/JN800306/poultry/Peru/1918-03/2008
XIII/JQ267579/chicken/Iran-EMM/7/2011
XIII/JX436341/chicken/Pakistan/MM15/Sheikhupura/2011
XIV/HF969141/chicken/Nigeria/NIE08-2359/2009
XIV/HF969155/chicken/Nigeria/NIE09-2087/2009
XVII/FJ772463/chicken/Burkina Faso/2415-580/2008
XVII/KF442614/chicken/Nigeria/228-7/2006
XVIII/HF969179/chicken/Ivory Coast/CIV08-026/2007
XVIII/HG326600/village weaver/Ivory Coast/CIV08-032/2006
VI/AB465606/chicken/Japan/Ibaraki/85/1985
VI/JN638235/dove/Italy/11RS100 104VIR/2011
VI/JN872177/chicken/USA/Massachusetts/344783-3/2004
VI/AF109885/domesticfowl/Great Britain/GB1168/84/1984
VI/JX901341/pigeon/USA/PA/USA/0712/2007
VI/KJ607164/pigeon/China/pi/CH/LJS/1/03/2003
V/JN941993/pelican/USA/Wisconsin/2415-02/2008
V/EU518684/chicken/Mexico/chiken/Mexico/466/2006
V/JQ217177/chicken/Kenya/A89/2010
VIII/AF048763/chicken/Malaysia/AF2240/1960
XVI/JX186997/chicken/Dominican/Republic/867/2008
XVI/JX915243/chicken/Mexico/Queretaro/452/1947
IV/AY741404/Fowl/UK/Herts/1933
XI/HQ266602/chicken/Madagascar/MG/725/08/2008
IIIGU182327/chicken/Pakistan/SPVC/Karachi/NDV/1/1974
IX/FJ436302/chicken/China/F48E8/1946-1948
I/M24693/chicken/Australia/Queensland/66/1966
I/GU332645/chicken/Australia/9809-19-1107/1998
I/KC503467/northernpintail/USA/AK/44493-801/2009
X/EF564826/northern pintail/US/OH/87-486/1987
II/GU978777/chicken/USA/(TX)GB/1948
II/AF077761/chicken/USA/Lasota/1946
I-VI
VIII-XVIII
0.02

Phylogenetic analysis

In order to confirm the evolutionary relationship between the NDV isolates studied here and viruses isolated in other geographical regions, a phylogenetic analysis was performed. Other isolates that were closely related to those studied in the present work were selected from the GenBank database and used for a final alignment. The dataset contained 82 complete F gene nucleotide sequences: sub-genotype VIId (n = 37) (including the 21 sequences obtained in this study), the rest of the sub-genotypes in genotype VII (n = 10) and selected isolates representing the rest of the currently classified genotypes of NDV class II (n = 35) (Table S1). A phylogenetic tree based on the complete coding sequences of the fusion protein gene of these 82 NDV isolates is presented in Figure 1. Expectedly, the isolates studied here clustered together with the viruses that showed highest nucleotide sequence identity to them. The fourteen isolates that showed the highest genetic similarity between them (Fig. 1, group 1) formed a monophyletic branch with the two isolates from wild birds in Serbia. They all grouped together with isolates obtained from poultry and wild birds mainly in China during 2001-2013, but also from chickens in Venezuela and Colombia in 2009. The other seven isolates (Fig. 1, group 2) clustered with duck/Russia/Adygea/12/2008 and turkey/Israel/832 497/2009, forming another monophyletic branch in the dendrogram.

Discussion

Here, we report the isolation and characterization of virulent Newcastle disease viruses isolated primarily from backyard chickens in Bulgaria and Ukraine and from a pigeon in Ukraine and a wild bird in Russia. Overall, this study allowed the recognition of circulation of vNDV of sub-genotype VIId in Bulgaria and Ukraine during 2002, 2003, 2006-2009, 2011 and 2013 and identification of the most closely related NDV isolates from Serbia and other geographical locations.

We provide here the first complete F gene sequences of NDV of sub-genotype VIId from Bulgaria and Ukraine, confirmed by multiple isolations. In contrast, previous reports describing Newcastle disease outbreaks have identified the circulation of genotypes IV, VI and VIIb in Bulgaria during the 1990s [1, 10, 16]. There were no reports on the genotype distribution of NDV in Ukraine prior to this study. However, partial F gene sequences of viruses of genotypes II and VIIb isolated in the 1990s were available in GenBank database (http://www.ncbi.nlm.nih.gov/genbank). Additionally, prior to 2002, sub-genotypes VIb, VIIa and VIIb were predominantly identified in neighboring Russia and the nearby country of Kazakhstan [7, 18, 36]. No virulent viruses of other genotypes were isolated in Bulgaria during the active surveillance in either commercial or backyard poultry holdings throughout the study period. Our results provide evidence that viruses of sub-genotype VIId replaced the strains that were prevalent in this country before 2002. This conclusion was supported by the results of partial F gene analyses of Bulgarian isolates identified between 2005 and 2007 showing them to be VIId isolates [14, 34]. Similar to our results, after 2002, viruses of sub-genotype VIId were also reported to replace the previously predominant isolates of sub-genotype VIIb in Kazakhstan [7]. Sub-genotype VIId is the predominant group of viruses responsible for most ND outbreaks in Asia since the end of the last century [19]. The more frequent identification of viruses of sub-genotype VIId in Eastern Europe is an example of the western spread of this group of viruses, as closely related strains were also isolated in Romania, Greece and Hungary [5]. Genotype VII represents a large and genetically diverse group of viruses and has been associated with recurrent poultry outbreaks, mainly in the Middle East and Asia [25]. Sporadic events in Africa and South America [1, 7, 40, 49] and the intercontinental spread of recently identified sub-genotype VIIi with panzootic potential [28] demonstrate the global significance and economic importance of these viruses.

Fourteen ND viruses from Bulgaria and Ukraine isolated during 2003, 2006-2009, 2011 and 2013 (Fig. 1, bold) showed a high level of genetic similarity among them and to isolates from wild birds in Serbia in 2007. Such high nucleotide sequence identity (99.3-99.7 %) is indicative of an epidemiological link between the outbreaks or introductions from a common source. These results suggests that these 14 NDV isolates (Fig. 1, group 1) of sub-genotype VIId from Bulgaria and Ukraine may have been part of a broader epizootic process in Eastern Europe rather than separate introductions. This hypothesis also corresponds to the conclusions of Vidanović et al. [46] and Dimitrov [14], as genetically similar viruses have also been recovered from poultry in Macedonia, Serbia and Turkey. The rest of the studied viruses (n = 7, Fig. 1, group 2) were less closely related to these 14 NDV isolates (97.3 % nucleotide sequence identity between the two groups). Considering that both groups of viruses (Fig. 1, groups 1 and 2) were circulating during the same time period, it is not likely that they evolved from each other. It is possible that they represent two separate introduction events of vNDV that evolved elsewhere or that they evolved locally from an unidentified common ancestor introduced earlier in the area.

The means by which these viruses have been maintained (up to 10 years between isolations) is puzzling. The possibilities of maintenance in partially immune poultry or in

disease resistant wild birds are viable hypotheses, as most backyard poultry do not receive vaccination. However, it has been suggested that vNDV may be maintained in vaccinated poultry [5, 8, 39], as post-vaccination immunity prevents clinical diseases but not shedding [24, 35]. There are two regularly documented natural reservoirs of virulent NDV (columbids and double-crested cormorants), but they harbor viruses of different genotypes (VI and Va, respectively) [15, 26] and are not likely to be the source of the viruses studied here. The role of other wild birds as a reservoir of virulent NDV of other genotypes is disputable, as evidence supporting this is scarce.

The virulent NDV isolates from Bulgaria, Ukraine and Russia studied here were genetically related to isolates from distant geographical locations in Asia, Israel and Russia. Furthermore, partial nucleotide sequence analysis (374 nt of the fusion protein gene coding sequence) showed a close genetic relationship (98%) of the viruses from Bulgaria and Ukraine to isolates from poultry from Saudi Arabia in 2000 and South Africa in 2004 (data not shown). These findings provide an indication of east-to-west and north-to-south transmission of vNDV. The close genetic relationship between isolates from Bulgaria and Ukraine and those from China, Israel, South Africa and Russia might be explained by migration of wild birds. It has been shown that wild birds play a role in the epidemiology of NDV [4, 47]. Anthropogenic factors such as trade and/or illegal movement of birds and poultry products cannot be excluded as mechanisms of introduction of the studied NDV strains. Although possible, we had no sufficient evidence to support any of these hypotheses. Furthermore, the isolates from wild birds from Serbia were linked to simultaneously ongoing poultry outbreaks in the area [46]. Nevertheless, the presence of ND viruses that are very closely related to isolates from distant geographic locations is supportive of the capacity for high mobility and widespread circulation of this group of viruses.

Overall, our study emphasizes the importance of investigating the epidemiology of NDV and studying the mechanisms for intercontinental spread of the virus. It also underlines the need of continuous monitoring of backyard poultry for the presence of circulating vNDV. Early identification of NDV in backyard poultry followed by recommended control measures [33] could help in the prevention of further spread of new genetic variants. Although vaccination practices help to control the disease in Bulgaria and Ukraine, as evidenced from the absence of outbreaks in industrial poultry holdings, widespread backyard poultry farming, where usually no vaccination is performed, poses a significant threat and provides opportunities for future ND outbreaks.

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